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ETHEL PERCY ANDRUS GERONTOLOGY CENTER
(213) 743-6060

September 27, 1989

Dr. Jeannine A. Majde
Code 1141SB
Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217

RE: N00014-89-J-1005 Annual Report

Dear Dr. Majde:

Enclosed are three copies of the Annual Progress Report for "Steroids, Stress, and Gene Expression in Neurons". If you need additional Information please contact me at (213) 743-5168.

Sincerely,

Caleb E. Finch, Ph.D.
Principal Investigator

and

Nancy R. Nichols, Ph.D.
Co-Principal Investigator

Connie Whitley
Sr. Contract and Grant
Administrator

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Dr. Jeannine A. Majde (3)
Code 1141SB
Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217

Mr. Robert Bachman (1)
University of California, San Diego
MC A-034
La Jolla, CA 92093-0234

Director Naval Research Laboratory (6)
Attention 2627
Washington D.C. 20375

Defense Technical Information Center (12)
Building 5
Cameron Street
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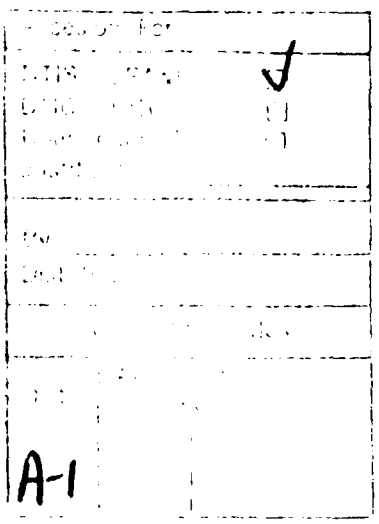

ABSTRACT

In order to identify glucocorticoid responses that mediate cellular adaptation to stress, we have examined changes in gene expression in rat hippocampus in response to acute corticosterone (CORT) treatment or to vibratory stress. Previously, we described select changes in four poly(A)-containing RNAs, coding for 50, 35, 33 and 20 kd polypeptides, in response to administered CORT. We have cloned five CORT-responsive mRNAs from a rat hippocampal cDNA library by differential hybridization and compared them to the previous responses by hybrid selection, in vitro translation, and 2-d gel analysis. Two of the clones hybridized to mRNAs coding for the 50 and 35 kd polypeptides; they were identified from partial DNA sequences as glial fibrillary acidic protein and glycerol phosphate dehydrogenase. Another clone was identified as glutamine synthetase and two were not identified, CR16 and CR62. CORT and stress responsiveness of these mRNAs were compared by 2-d gel analysis of in vitro translation products or RNA hybridization experiments. Only GPDH/35 kd and 20 kd were responsive to 2 hr CORT treatment and 2 hr vibratory stress. The lack of response to stress of the other mRNAs, despite elevated CORT levels, is consistent with their slower response to exogenous CORT. One exception is 33 kd which does respond to 2 hr CORT treatment but not to 2 hr vibratory stress. Therefore, we find subsets of mRNA responses which differ in temporal and other aspects of CORT regulation. Examining a variety of stressors and chronic stress will determine if this is a general or unique pattern of expression of CORT-dependent responses in hippocampus.

INTRODUCTION

Glucocorticoids mediate cellular adaptation to stress by a variety of mechanisms including receptor-mediated changes in gene expression (Munck et al., 1984). Most studies in brain have focussed on neuropeptides or peptide hormones that regulate the hypothalamic-pituitary-adrenal (HPA) axis and hence the output of glucocorticoids (Swanson et al., 1986; Dallman et al., 1987). Additional mechanisms for adaptation to stress might be revealed by examining patterns of gene expression in brain in response to elevated circulating glucocorticoid levels. Since the hippocampus is both a glucocorticoid target tissue with a high concentration of receptors and a site of negative feedback regulation of the hypothalamic-pituitary-adrenal axis (McEwen et al., 1986; Ratka et al., 1989), we have examined changes in gene expression in rat hippocampus in response to corticosterone (CORT) and physiological stress.

Previously, we described select changes in four poly(A)-containing RNAs, coding for 50, 35, 33 and 20 kd polypeptides, in response to administered CORT (Nichols et al., 1988a). In addition, we have cloned five CORT-responsive mRNAs from a rat hippocampal cDNA library by differential hybridization (Masters et al., 1987; Nichols et al., in press). In this report we characterize (and in some cases, identify) the cloned mRNAs and compare them to the previous mRNA translation products. In addition, we have analyzed these CORT-responses for responsiveness to 2 hr vibratory stress. Based on these data, we discuss how these molecular markers may be used to investigate mechanisms of cellular adaptation to stress.

METHODS

Animals, Treatments, RNA Isolation and CORT RIA

Male Fischer 344 rats (200-250 g) were multiply housed with a controlled light-dark cycle (lights on 06.00 h and off 18.00 h). Food and water were ad libitum. Rats were either adrenalectomized (ADX) and maintained on 0.9% saline or intact; ADX rats were injected subcutaneously with CORT or Mazola corn oil vehicle. Following decapitation (2 or 8 h after a morning injection or 2 h after initiation of stress), hippocampi were rapidly dissected and stored at -80°C until total RNA was isolated from pooled or individual samples by a guanidinium thiocyanate/CsCl procedure (Nichols et al., 1988a). Trunk blood was collected for measurement of serum CORT by radioimmunoassay (Nichols et al., 1989).

Isolation and Identification of Rat Hippocampal cDNA Clones

A rat hippocampal cDNA library was constructed in λgt10 from 1 μg of poly(A)-containing RNA from both ADX and ADX + CORT (10 mg/day for 3 days) groups. Approximately 100,000 plaques were screened on replicate nitrocellulose filters by differential hybridization using ^{32}P-cDNA prepared from 0.5 μg of each poly(A)-containing RNA pool used to make the library. CORT-responsive cDNA clones were rescreened twice, subcloned into the Bluescribe or Bluescript vectors (Stratagene) and partial DNA sequences were compared with known genes in GenBank (Nichols et al., in press).

Hybrid Selection, In Vitro Translation and Two-dimensional Gel Analysis

Rat brain mRNAs were selected from total RNA using immobilized cDNA clones and the hybrid selection method of Mason and Williams (1985). Total RNA or select RNA was used as a template for in vitro translation by a rabbit reticulocyte system with ^{35}S-methionine (Nichols et al.,

in press). Translation products were resolved by two-dimensional (2-d) gel electrophoresis, visualized by fluorography and quantitated by computerized videodensitometry (Nichols et al., 1988a).

RNA Hybridization Analysis

Total RNA from treated and control individual or pooled samples were either fractionated on formaldehyde (0.2M)/agarose gels and transferred to positively charged nylon membranes (Nichols et. al., in press) or vacuum-blotted directly on nylon membranes using a slot-blot apparatus. Anti-sense ^{32}P-cRNA probes were transcribed from linearized subclones in a plasmid vector and random-primed ^{32}P-cDNA probes were made from gel-purified inserts. Hybridization procedures and quantitation of autoradiographs were as described in Nichols et al. (in press). Sizes of transcripts were determined by comparison with an RNA ladder stained with ethidium bromide. For more precise quantitation, RNA was titrated by the solution hybridization/RNase protection method of Lee and Costlow (1987) and expressed as pg transcript/μg total RNA.

RESULTS

We have used two methods to examine altered patterns of gene expression in rat hippocampus in response to administered glucocorticoids: analysis of RNA in vitro translation products on 2-d gels (Nichols et al., 1988a) and cloning of cDNAs from a rat hippocampal library by differential hybridization (Nichols et al., in press). We have further characterized CORT-responsive cDNA clones in hybrid selection/in vitro translation experiments and compared physico-chemical characteristics of the polypeptide products (and in two cases, co-migration on 2-d gel fluorographs) with previously characterized CORT-responsive mRNA translation products (Table 1). From these data and DNA sequence analysis (Table 1), two of the four mRNA translation products have been identified as glycerol phosphate dehydrogenase (GPDH)/35 kd and glial fibrillary acidic protein (GFAP)/50 kd; two others are still unidentified, 33 kd and 20 kd. An additional clone was identified as glutamine synthetase (GS/43 kd) and two were not identified, CR16 (not resolved on 2-d gels) and CR62/43 kd.

Based on their rapid response (2 h) of large magnitude (5- to 50-fold) to high doses of CORT (50 mg/kg/day) and type II glucocorticoid receptor-specificity, we postulated that the hippocampal mRNA increases should also occur during response to stress-induced elevations of glucocorticoid (Nichols et al., 1988a). Subsequently, we showed that similar increases in two of the three hippocampal mRNAs are elicited by the physiological stress of shaking for 2 h in a cage mounted on a metabolic shaker (Nichols et al., 1989). More recently, we have extended these studies to the cloned cDNA CORT-responses: GPDH/35 kd as an oligodendrocyte marker; CR16 as an unidentified gene expressed in hippocampal neurons; and proteolipid protein (PLP) as a non-changing control (Masters et al., 1987). Fig. 1 shows the results of RNA titration experiments on hippocampal total RNA prepared from individual rats in three treatment groups with the following serum CORT levels (ng/ml): ADX + stress, <10; intact, <10 - 51; intact + stress, 210 $\pm$ 40. Although both GPDH and CR16 mRNAs increase in response to CORT in a dose-dependent manner and show type II glucocorticoid receptor specificity (unpublished data; Masters et al., 1988), they differ in their response to stress. In contrast to the significant 3-fold increase in GPDH mRNA abundance after 2 h vibratory stress, the CR16 transcript does not change between intact and intact-stressed rats. However, CR16 unlike GPDH mRNA does change significantly between ADX (not shown) or ADX-stressed and intact rats with low A.M. levels of CORT.

In RNA slot blot hybridization studies, GFAP and GS mRNAs also did not respond to 2 h vibratory stress (not shown). The data collected to date on CORT and stress responses are summarized in Table 2. Only GPDH/35 kd and 20 kd were responsive to both 2 h CORT treatment (1 mg and 10 mg, respectively) and 2 h vibratory stress (Nichols et al., 1989). The lack of response to stress of the other RNAs, despite elevated CORT levels, is consistent with their slower response to exogenous CORT (>2 h but <8 h). One exception is the RNA coding for the 33 kd polypeptide, which does respond to 2 h CORT treatment but not to 2 h vibratory stress.

Although GFAP/50 kd did not respond to physiological elevation of CORT following 2 h vibratory stress, we have recently demonstrated that the gene is under physiological regulation by CORT, since it is disinhibited after 7 days (but not 3 days) ADX (Fig. 2). This response is similar albeit in the opposite direction to the results observed for CR16 mRNA where the difference between A.M. intact levels and ADX occurs as early as 1 day post-ADX (unpublished data). As expected, GFAP mRNA decreases within 8 h after CORT replacement in ADX rats (Nichols et al., in press).

CONCLUSIONS

We have demonstrated by hybrid selection of rat brain total RNA with CORT-responsive cDNA clones and subsequent in vitro translation/2-d gel analysis that two of the clones hybridize to mRNAs coding for the 50 and 35 kd polypeptides. Partial DNA sequences of both clones and comparison to known genes in Genbank have identified them, and hence their translation products, as GFAP and GPDH, respectively (Table 1). The RNAs coding for the 33 and 20 kd polypeptides remain unidentified. A translation product was not resolved for CR16 on 2-d gels with a pI gradient of 5.0 to 7.0, which is consistent with its extremely basic isoelectric point, predicted from translated coding region DNA sequence (unpublished data).

We have found three subsets of hippocampal mRNA responses to CORT and vibratory stress which differ in temporal and level-dependent aspects of CORT regulation (Figs. 1 and 2 and Table 2). Adrenal medullary cells release catecholamines and substance P differentially in response to two different stressful circumstances (Vaupel et al., 1988). However, several different stressors increase preproenkephalin A mRNA in rat hypothalamus (Yoshikawa et al., 1985; Lightman and Young, 1987). Examining a variety of stressors, chosen to differ in terms of CORT levels attained and activation pathways, will determine if the data in Table 2 represent a general or unique pattern of expression of CORT-dependent responses in hippocampus.

Additional experiments on kinetics of response, circadian variation, and chronic stress are also warranted in order to interpret differential stress reponse data. For example, no difference between ADX and A.M. intact levels of GPDH mRNA (Figs. 1) is consistent with GPDH being regulated solely by type II glucocorticoid receptors as a molecular stress response (Nichols et al., 1988b). Since low A.M. CORT levels significantly alter GFAP and CR16 expression (Figs. 1 and 2), they may also be regulated by type I receptors and modulate stress responsiveness (Masters et al., 1989; Ratka et al., 1989). Mechanisms of adaptation to stress in brain are likely to involve responses that are both mediated by glucocortcoids and opposed by them (McEwen and Brinton, 1987). In this context, elevation of GFAP is an early astrocytic response to brain injury; a decrease

in GFAP mRNA and protein (O'Callaghan et al., in press) by CORT may be a homeostatic, adaptive response (Munck et al., 1984; Nichols et al., in press).

In summary, these studies demonstrate the feasibility of using sensitive probes to examine changes in gene expression in hippocampus in response to hormonal and environmental stress. By examining their temporal cascades and altered patterns of expression, we may identify mechanisms by which the brain coordinates acute and chronic adaptive responses to stress.

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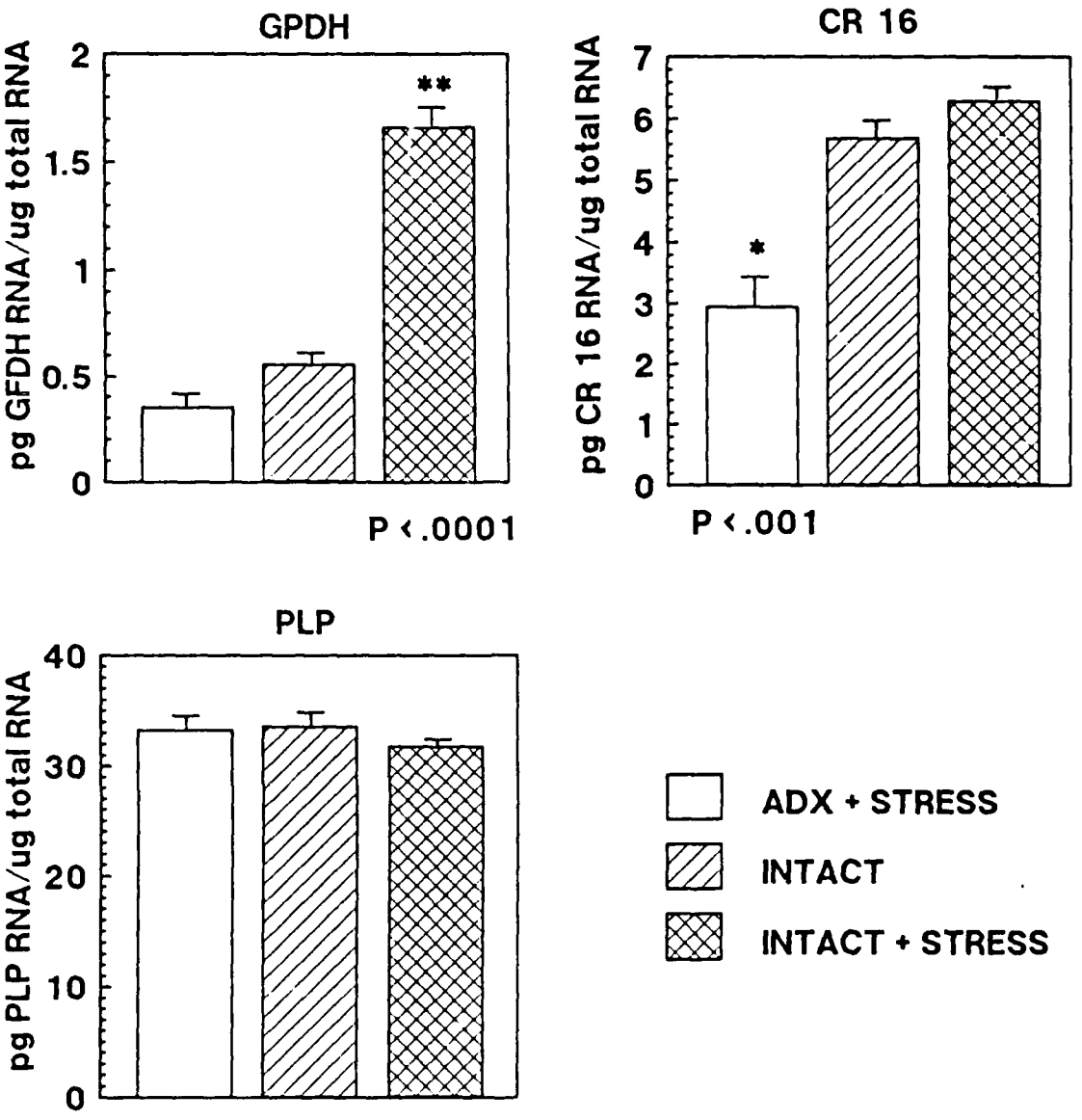

Fig. 1. Hippocampal total RNA was isolated from individual intact non-stressed rats or intact and ADX rats which were given vibratory stress for 2 h in a cage mounted on a shaker. For RNA titration experiments, an excess of ^{32}P-labelled antisense RNA synthesized from subcloned cDNA inserts was hybridized to increasing concentrations of total RNA, followed by RNase A and T1 digestion and acid precipitation. Changes in the abundance of GPDH, CR16 and PLP RNAs in individual rats of each treatment group were calculated from the slopes of titration curves. The mean $\pm$ SE are shown as histograms and significance levels (ANOVA) are versus non-stressed intacts.

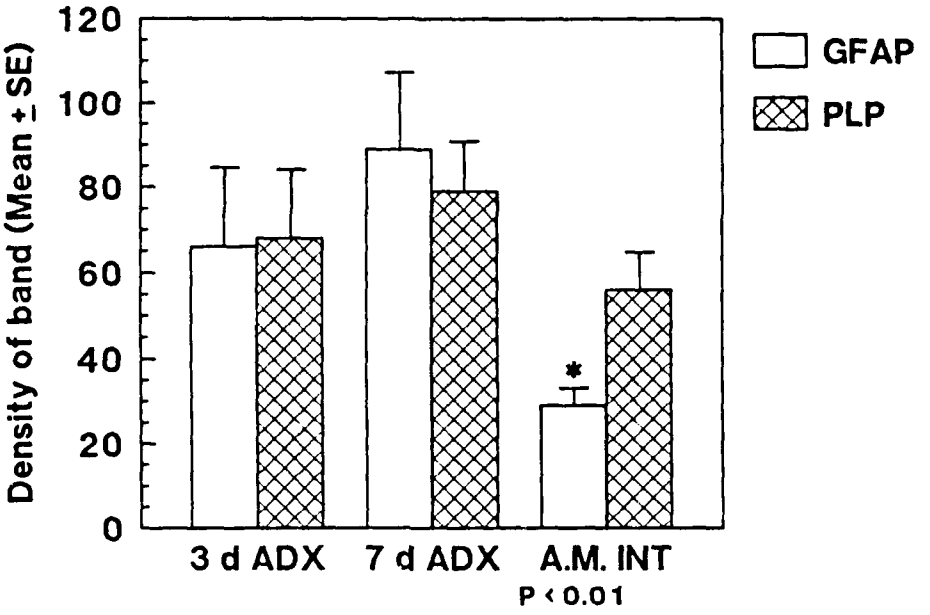

Fig. 2. Hippocampal total RNA was isolated from individual rats that were intact or ADX for 7 or 3 days and sacrificed in the morning. RNA samples (1 μg/lane) were size fractionated on formaldehyde/agarose gels, transferred to nylon and hybridized with an antisense rat GFAP or PLP ^{32}P-cRNA probe. The total integrated density of a band was determined by computerized densitometry with automatic background subtraction. The mean $\pm$ SE are shown as histograms and significance levels (ANOVA) are versus intacts.

Table 1

IDENTIFICATION OF CORT-RESPONSIVE CLONES/TRANSLATION PRODUCTS

CLONE	GENE	TRANSLATION PRODUCT MOL. WT.	pI
CR 1	GLUTAMINE SYNTHETASE	43 kd	6.7
CR 3	GLYCEROL PHOSPHATE DEHYDROGENASE	35 kd*	6.3
CR 16	UNKNOWN	NOT FOUND	
CR 46/59	GLIAL FIBRILLARY ACIDIC PROTEIN	50 kd*	5.5
CR 62	UNKNOWN	42 kd	5.4
	UNKNOWN	33 kd*	6.5
	UNKNOWN	20 kd*	6.9

*Previous CORT-responsive RNA translation products (Nichols et al., 1988)

Table 2

SUMMARY OF DIFFERENTIAL RESPONSES TO STRESS

RESPONSE	FOLD-CHANGE (TMT/CONTROL)	
	2 HR CORT	2 HR VIBRATORY STRESS
GPDH/35 kd	3	3
20 kd	10	3
33 kd	27	1
CR 16	1	1
GFAP/50 kd	1	1
GS/43 kd	1	1